Molecular Characterization And Ecotoxicological Evaluation of The Natural Dye Madder And Its Chlorinated Products

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Abstract

There has been increased interest in the use of natural dyes for textile coloration as alternatives to synthetic dyes, due to the general belief that natural dyes are more environmentally friendly. However, natural dyes have poor affinity for textiles, which can lead to high dye levels in the resultant wastewater. While chlorine treatment has proven to be effective for dye wastewater disinfection and decolorization, this process can also lead to the formation of more toxic degradation products for certain synthetic dyes. On the other hand, little information is available regarding the ecotoxicity of natural dyes and their chlorination products. To advance knowledge in this area, madder was selected due to its historical importance and wide application in the textile industry. Specifically, we sought to characterize the chlorine-induced degradation products of an aqueous madder solution and to assess their ecotoxicity. The main component of the present madder sample was Alizarin (89.8%). Chlorination led to complete decolorization, and 2-hydroxynaphthalene-1,4-dione and phthalic anhydride were identified as key degradation products. Chlorination of madder decreased toxicity to *Daphnia similis* (microcrustacean) 10-fold and removed the toxicity to *Raphidocellis subcapitata* (algae), when compared to the parent dye.

Introduction

Natural dyes were the only source of color for textiles until the serendipitous formation of the dye Mauveine by William Perkins, in 1856 (Drivas et al. 2011). Following this discovery, synthetic dyes soon became available worldwide, and by 1880 most natural dyes were replaced by synthetic dyes on a global scale (Drivas et al. 2011; Yusuf et al. 2017). This occurred because natural dyes had the disadvantage of needing a metal (Met) mordant (e.g., Al^{3+}, Fe^{2+}) for anchoring them to textile fibers (cf. Figure 1A), due to their generally poor affinity for commercially available fibers. However, commercial synthetic dyes exhibited direct fiber affinity (cf. Figure 1B, high reproducibility of their shades, wide range of colors, and more cost-effective application methods (Samanta and Konar 2011).

In the early days of synthetic dyes, concerns about their use were mainly related to performance and costs, as the industrial community was not yet aware of the potential adverse effects of dyes on human health and aquatic life (Dharma Trading 2017). Over time, the accumulation of data pertaining to the toxicological properties of certain synthetic dyes and their precursors to a variety of organisms (Bandala et al. 2008; Erkurk et al. 2007; Gao and Tan 2013) led to renewed interest in the use of natural dyes as alternatives to synthetic ones. Unfortunately, the fact that natural dyes have poor affinity for textiles (Samanta and Konar 2011) can lead to high dye levels in the resultant wastewater and the need for treatment to remove them (Chequer et al. 2013).

Dyes reaching wastewater treatment plants can undergo partial or complete transformation/degradation of the parent compound. When degradation is incomplete, reaction products generated can have different toxicological properties than the parent compound (Rodil et al. 2012; Puvaneswari et al. 2006; Copaciu et al. 2013). One of these treatment methods is chlorination (Cl_2 treatment), which is the best known and most common method used worldwide in wastewater treatment plants for water disinfection purposes.

Chlorine can be applied as a gas which is cost-effective and efficient (Drinan and Spellman 2013; Stuetz and Stephenson 2009). For certain azo dyes, chlorination of wastewater has also led to the formation of toxic (Vacchi et al. 2013) and mutagenic compounds (Vendemiatti et al. 2021; De Oliveira et al. 2006; Watanabe et al. 2001).

Among the natural dyes used in textile coloration is the well-known orange-red dye madder, found in dried roots of the *Rubia tinctorum L* plant. Madder has been used since ancient times in the coloration of silk, wool and cotton, producing shades from pink to black, depending on the mordant used (Ferreira et al. 2004). Its composition depends on the species from which extraction occurred and the extraction method employed (Bechtold and Massak 2009); however, it is generally composed of anthraquinone derivatives. There are several studies concerning madder's carcinogenic and mutagenic potential (Ino et al. 1995; Inoue et al. 2008a; Inoue et al. 2008b; Inoue et al. 2009; Yasui and Takeda 1983; Westendorf et al. 1988; Jäger et al. 2006; Kawasaki et al. 1992), but no information was found concerning the aquatic toxicity of madder or its chlorinated solutions.

The objective of this study was to characterize a madder sample, treat it with chlorine gas, and study the composition and the ecotoxicity of the resultant chlorinated solution. Additionally, we wanted to propose a degradation pathway for the major component of the studied madder sample after chlorination.

Materials And Methods

Madder was obtained from the dye inventory of the Wilson College of Textiles at North Carolina State University and alizarin (97%) was purchased from Sigma Aldrich. Both were used without further purification.

2.1. Decolorization of madder solutions

A 100 mg/L aqueous madder solution was treated with chlorine gas generated from the dropwise addition of 20 mL of 12 M HCl to 3 g of potassium permanganate ($KMnO_4$). Color removal was monitored by UV-Visible spectroscopy (Vacchi et al. 2013) and the amount of residual free chlorine was determined using the N,N'-diethyl-*para*-phenylenediamine (DPD) method (American Public Health Association 1995).

2.2. Analysis of madder solutions

Madder solutions before and after chlorination were analyzed using HPLC-DAD, TLC, and mass spectrometry. HPLC-DAD was performed using a Waters C_{18} column (150 x 3.9 mm), a flow rate of 1 mL/min, and an injected volume of 10 µL. Solvents used were 0.1% aqueous trifluoroacetic acid (TFA) and acetonitrile (ACN) in a gradient elution: 0–3 min: 15% ACN, 3–10 min: 15–45% ACN and 10–15 min: 45% ACN.

For TLC analysis, madder and its chlorinated solution were dissolved in methanol and spotted on silica gel 60 plates. The eluent used was chloroform/methanol/water (65:30:5), and visualization of TLC components was performed using a UV lamp.

Mass spectrometric analysis of madder was carried out using a high-resolution mass spectrometer (Thermo Fisher Scientific Exactive Plus MS, a benchtop full-scan Orbitrap™) using Heated Electrospray Ionization (HESI). Samples were analyzed via LC injection into the mass spectrometer at a flow rate of 250 µL/min. The mobile phase B was acetonitrile containing 0.1% formic acid, and mobile phase A was water containing 0.1% formic acid. The mass spectrometer was operated in negative ion mode. Gradient elution was employed as follows: 0–3 min: 15% B, 3–10 min: 15–45% B, 10–15 min: 15% B, and 15–20 min: 15% B. HESI source parameters were: spray voltage 3.5 kV, capillary temperature 350°C, heater temperature 300°C, and S lens RF level 70 V, sheath gas flow rate 60, resolution 70,000 and scan range 50–750 m /z.

The chlorinated solution was analyzed using an Agilent 6520 Accurate-Mass-Q-TOF LC/MS spectrometer using Electrospray Ionization (ESI) in positive and negative mode. Instrument parameters were: gas temperature 350°C, drying gas 10 L/min, nebulizer 30 psi, fragmentor voltage 175 V, capillary voltage 3500.

2.3. Ecotoxicity evaluation

Toxicity of madder and its chlorinated solution was evaluated using the microcrustacean *Daphnia similis* and the algae *Raphidocellis subcapitata*. Chlorinated solutions were tested after no residual free chlorine was detected by the DPD method. The madder stock solution (100 mg/L) was prepared in water containing 0.1% DMSO to aid dissolution.

Acute toxicity to *D. similis* was performed according to OECD 202 guidelines (OECD 2004). Concentrations of chlorinated dye solutions were expressed in mg equivalents of madder/L. In each replicate (4 replicates total), five 6-24h old neonate organisms were exposed to the dye for 48h, at 20°C ± 2°C, with a light intensity of 1000 lux under the photoperiod (16/8h light/dark). Negative and solvent controls were included. Organisms were not fed. After a 48-h exposure period, immobilized organisms were counted. The tests were considered valid if the immobilization rate was less than 10% in the negative control group. Results were statistically analyzed for estimating the dye concentration leading to the immobilization of 50% of the organisms, EC_{50} (Hamilton et al. 1977).

For the algae *Raphidocellis subcapitata* toxicity test, three replicates of each concentration of madder and chlorinated madder solutions were analyzed. Solvent controls were included. The amount of algal biomass added was calculated according to the OECD 201 guidelines (OECD 2011). After the 72h exposure period, at 22°C ± 2°C, with a light intensity of 1000 lux, the number of cells was counted. Each test was considered valid if cellular growth in the control solution was at least 16-fold. The endpoint IC_{50} (concentration leading to growth inhibition rate of 50%) was determined statistically using analysis of variance.

Results And Discussion

3.1. Characterization of madder and its chlorinated solution

Results from HRMS analysis of the present madder dye sample indicated the presence of alizarin (*m/z* 239.03482) and purpurin (*m/z* 255.02980) as the major components (**0**), with 89.8% of the sample consisting of alizarin. Chlorination rapidly promoted dye decolorization, as evidenced by the disappearance of the absorption band centered at 430 nm (**0A**). HPLC-DAD and MS results (**0B**) show the disappearance of the peak corresponding to alizarin, retention time (r_t) 11.85 min.

The corresponding TLC chromatogram showed 5 main components (R_f = 0, 0.27, 0.53, 0.55 and 0.93) under UV light, Fig. 4. The ESI mass spectrum in Fig. 5 further confirmed degradation of alizarin, as its characteristic peak (m/z 239.0334), Fig. 5A, is no longer observed in the chlorinated solution (Fig. 5B). These observations are consistent with studies reported in the literature, which showed the loss of the anthraquinone chromophore during Cl_2-based treatment (Dušek and Kočanová 2016; Rajkumar et al. 2007).

Phthalic anhydride (*m/z* 149.0229) and 2-hydroxynaphthalene-1,4-dione (*m/z* 173.0249), **0A** and **6B** respectively, were identified as two of the Cl_2-induced degradation products. ESI/MS analysis of the individual TLC components showed the peak with *m/z* 257.0445 (Fig. 6C) for the fraction with R_f = 0.93. The molecular formula was $C_{14}H_{10}O_5$ and the corresponding structure is also shown in Fig. 6C. The results obtained suggest that for the exposure time evaluated in this work (60 min), Cl_2 promotes decolorization mainly by cleaving various C—C bonds of alizarin rather than by inserting Cl-atoms into the molecule through addition or substitution reactions.

Considering the degradation products identified in this work and in prior studies involving the decoloration of synthetic anthraquinone dyes of the type shown in Fig. 7 (Dušek and Kočanová 2016), decolorization of alizarin appears to involve a hydrolytic ring opening reaction to generate the corresponding dihydroxyarylketo-acid (cf. Figure 6C). Later in the process, phthalic anhydride forms as a key product, upon reversal of the acylation reaction associated with keto-acid structure formation. These two steps are illustrated by the retrosynthetic process shown in Fig. 8.

3.2. Ecotoxicity evaluation

There was a 10-fold decrease in acute toxicity to the microcrustacean *D. similis*, upon chlorination. EC_{50} was 4.4 mg/L (4.1–4.7, 95% confidence interval) for madder and EC_{50} 45 mg equivalent/L (39–53, 95% confidence interval) for its chlorinated solution, Error! Reference source not found.**9**.

Additionally, chlorination removed dye toxicity to algae *Raphidocellis subcapitata*. A maximum concentration of 10 mg/L chlorinated madder solution was tested, due to the dye's solubility properties and dilution levels required with algae growth media. The IC50 was 8.9 (± 0.4) mg/L calculated with the

results presented in Table 1. No inhibition in cell growth was observed for the chlorinated solution under the tested conditions.

Table 1. Number of algae cells observed from each concentration tested for the madder and chlorinated madder solutions.

Concentration (mg/L)	Madder		Chlorinated Madder	
	Average number of cells (10^4 cel/mL)	Statistical significance	Average number of cells (10^4 cel/mL)	Statistical significance
0.0[a]	341.0	ns	282.0	ns
0.1	275.7	ns	260.7	ns
0.5	294.7	ns	223.7	ns
1.0	276.7	ns	221.7	ns
5.0	291.0	ns	254.3	ns
10.0	139.7*	*	254.7	ns

[a]Corresponds to a solution of 0.1% of DMSO; ns: no significant difference from the control; * statistically significant ($p > 0.05$).

Conclusions

UV-Vis, TLC, HPLC-DAD, and LC-MS analyses were key in characterizing the components of a commercial sample of natural dye Madder (principally 1,2-dihydroxy-anthraquinone) and the products from its Cl_2-mediated decolorization. Multiple degradation products were produced during chlorination process and the major three were identified as non-chlorinated compounds comparable to those arising from certain commercial 1,4-diamino-substituted synthetic anthraquinone dyes. Chlorination of madder decreased toxicity to *Daphnia similis* (microcrustacean) 10-fold and removed the toxicity to *Raphidocellis subcapitata* (algae), when compared to the parent dye.

Declarations

Acknowledgments

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A. Ethics approval and consent to participate

Not applicable

B. Consent for publication

Not applicable

C. Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

D. Competing interests

The authors declare that they have no competing interests.

E. Funding

Conselho Nacional de Desenvolvimento Ciêntifico e Tecnológico – CNPq provided scholarship support for Tuane Santos and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Brasil, CAPES) – Financial Code 001 provided support for Francine Vacchi and Gisela Umbuzeiro, during the course of this study. Neither agency had a role in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript.

F. Authors' contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Tuane Santos, Yufei Chen, Josiane Vendemiatti, Adria de Oliveira, and Francine Vacchi. Harold Freeman, Nelson Vinueza, and Gisela Umbuzeiro provided project oversite for dye chemistry, mass spectrometry, and toxicology components, respectively. The first draft of the manuscript was written by Tuane Santos and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Figures

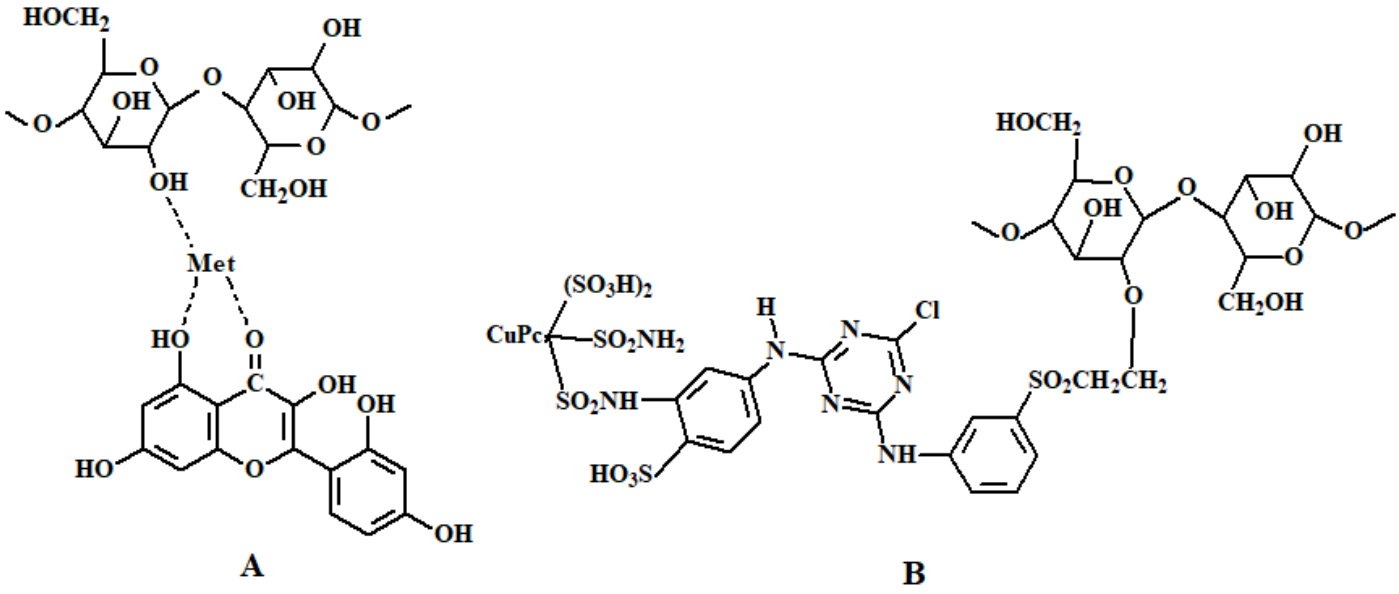

Figure 1

Illustration of natural dye (A) and fiber reactive synthetic dye (B) bonding to cellulose.

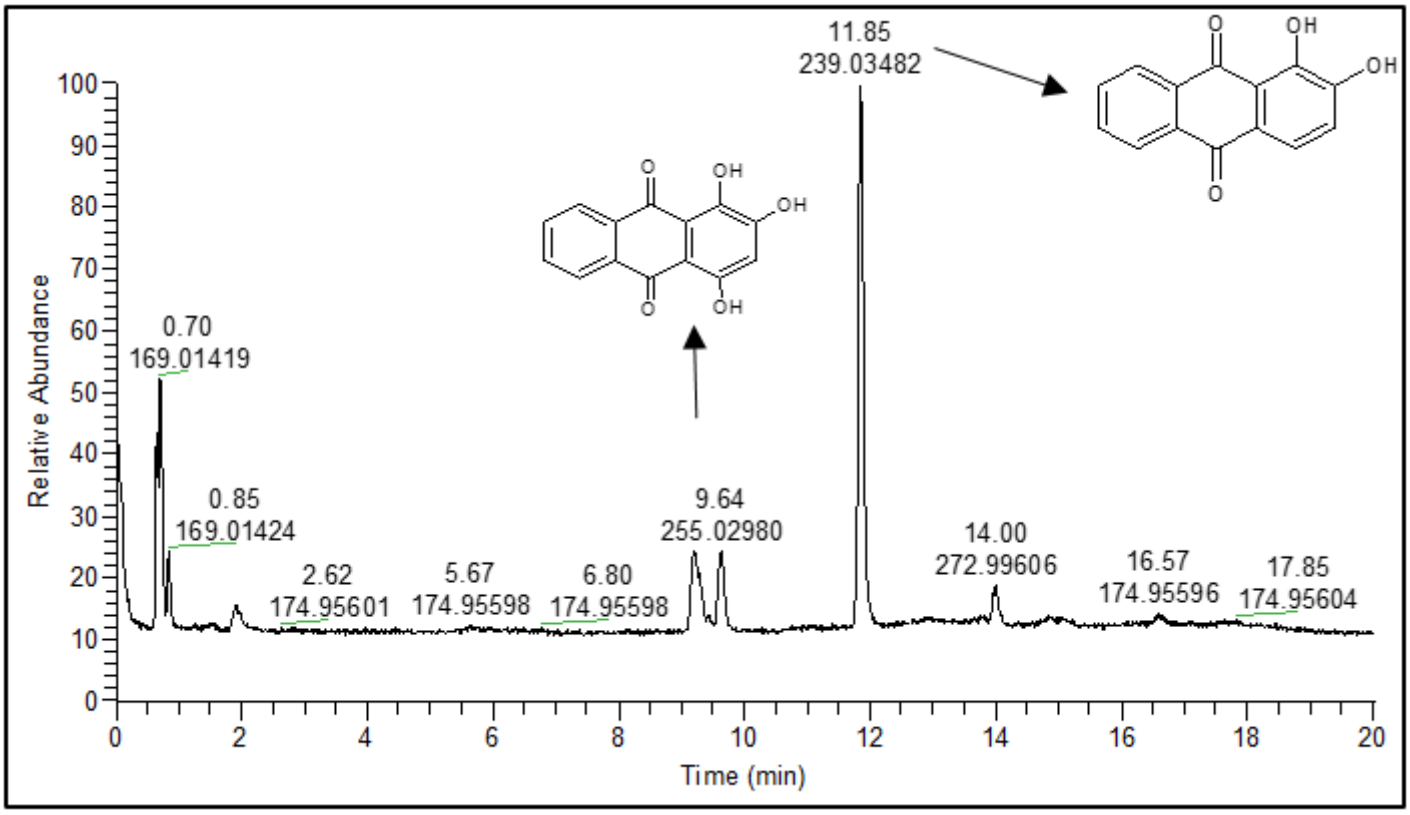

Figure 2

LC-HESI(-)-MS chromatogram obtained from the madder sample used in this study.

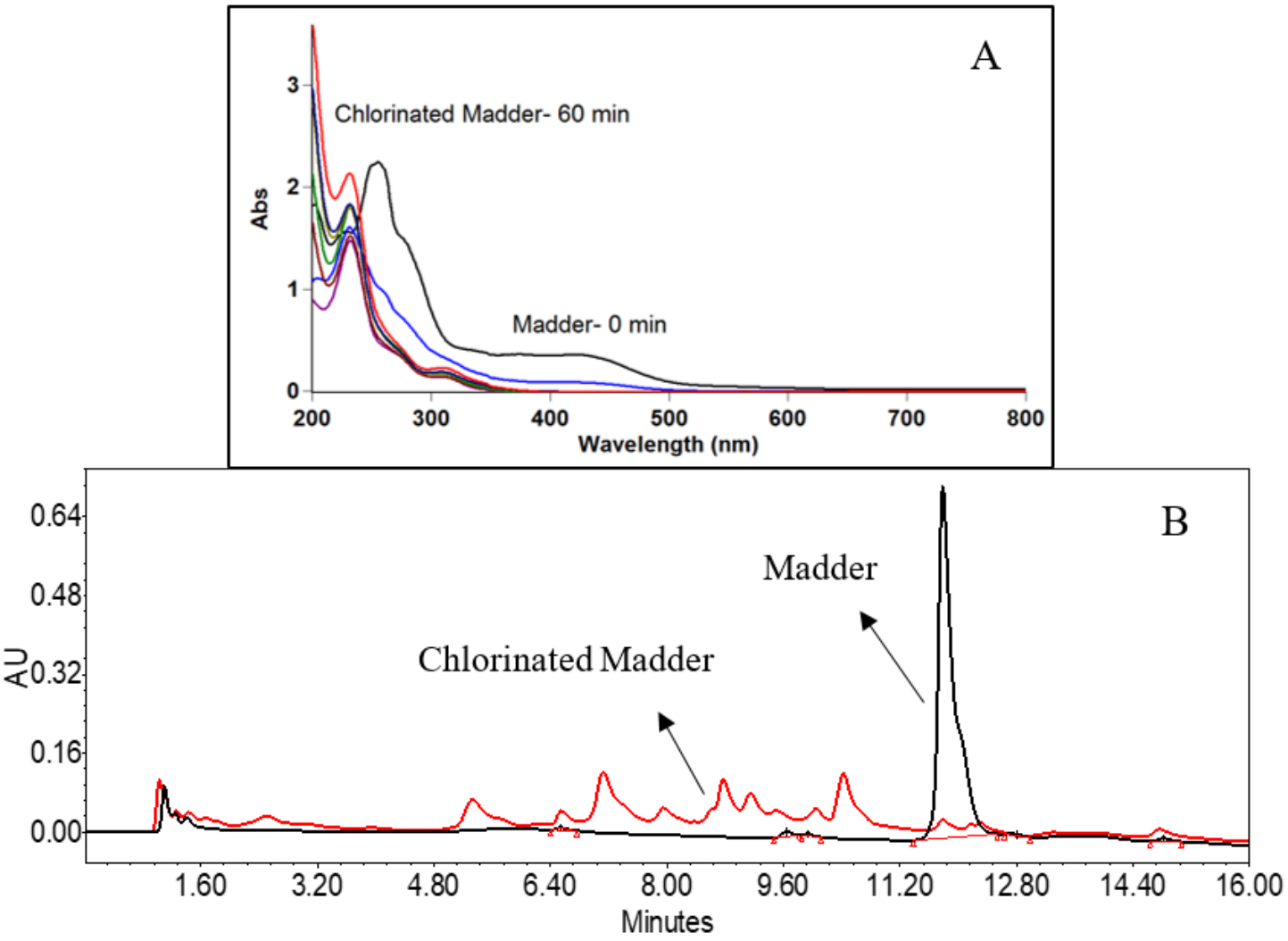

Figure 3

(A) UV-Visible spectra and (B) chromatograms from HPLC-DAD analysis of madder solutions before and after chlorination.

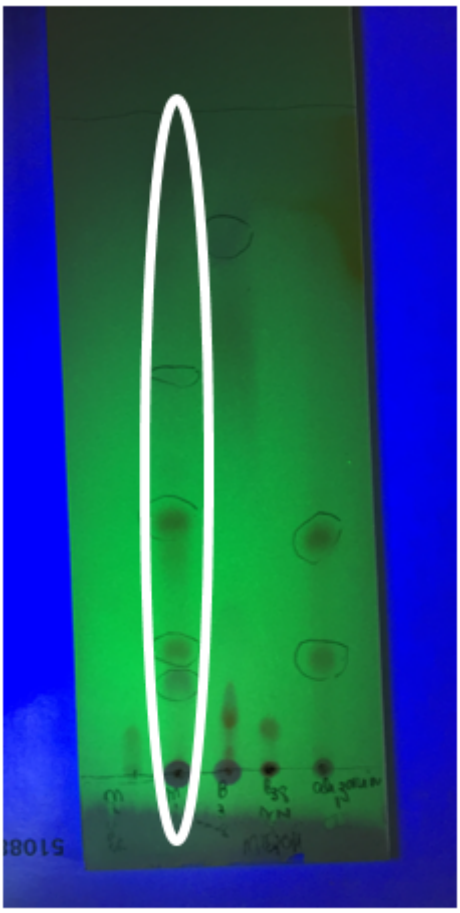

Figure 4

TLC analysis of chlorinated madder solution (circled) separated on silica gel using chloroform/methanol/water (65:30:5) eluent, detection under UV light.

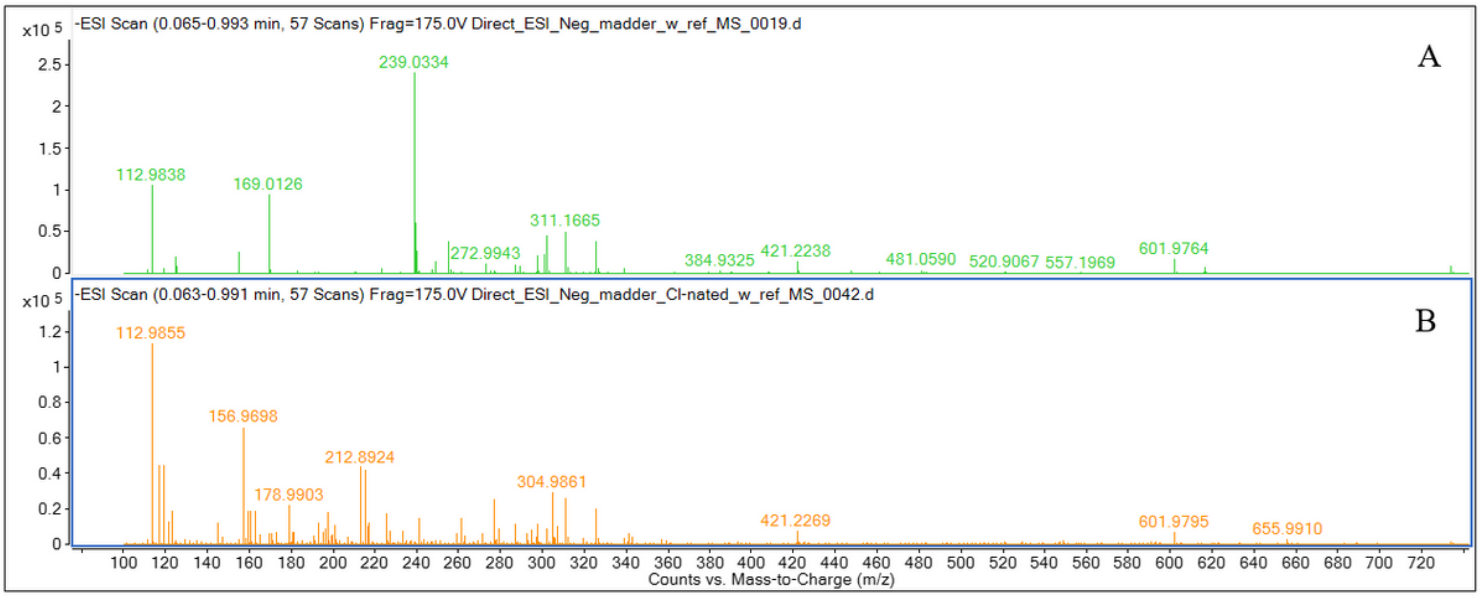

Figure 5

ESI(-)/MS spectra comparing (A) madder (m/z 239) and (B) its chlorinated solution.

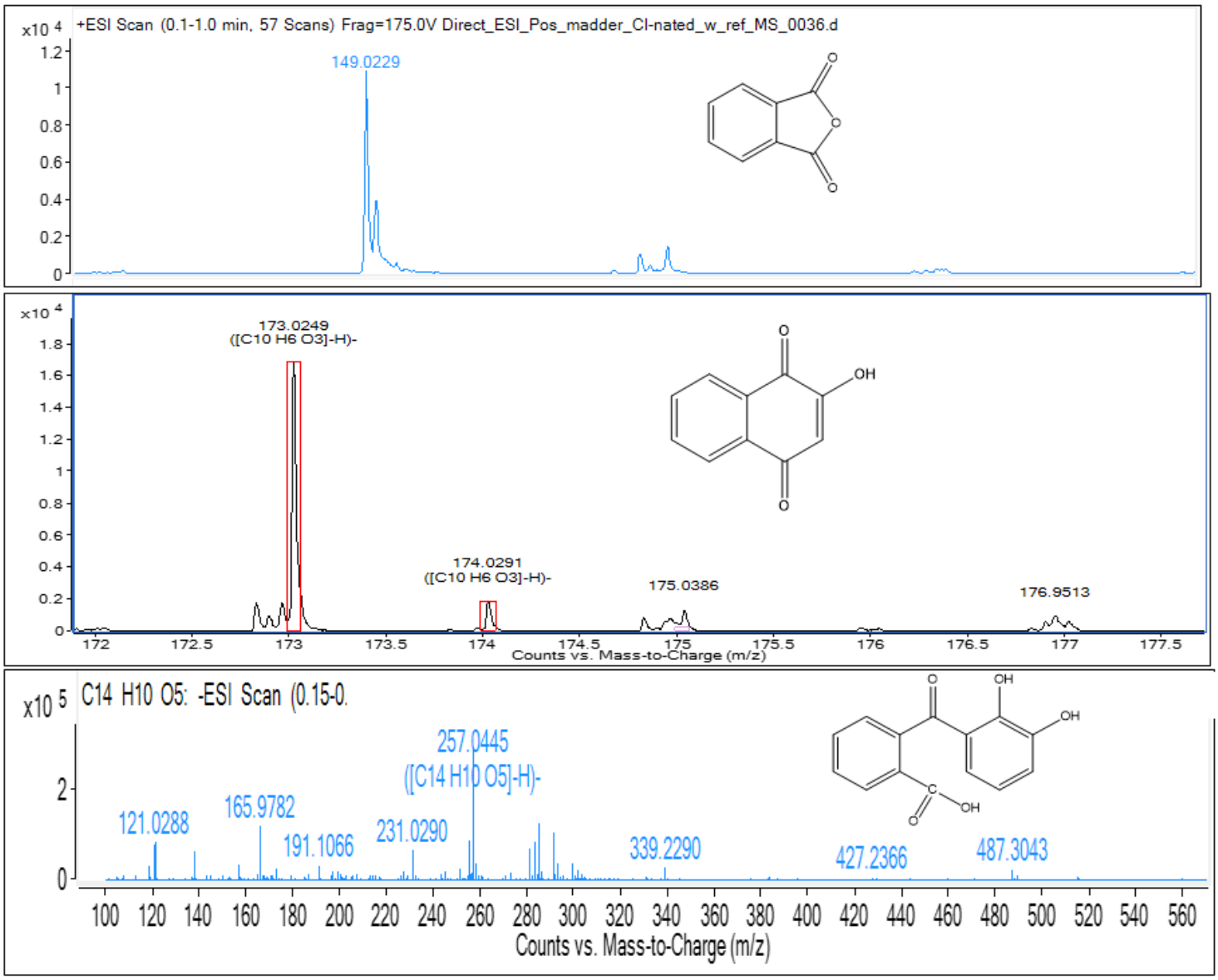

Figure 6

ESI/MS spectra showing identified degradation products (A) phthalic anhydride, (B) 2-hydroxynaphthalene-1,4-dione, (C) ring-opened product with m/z 257.0445, obtained from madder exposure to Cl2.

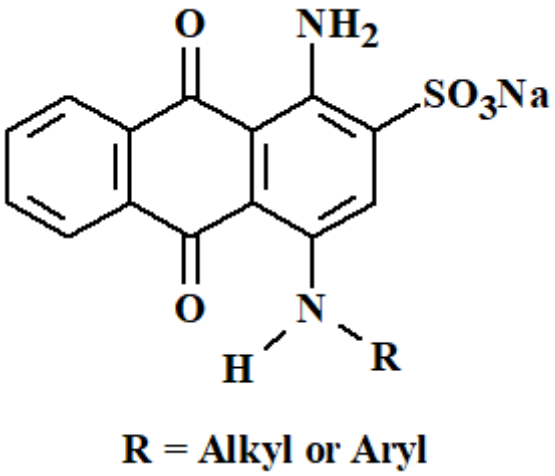

Figure 7

Examples of synthetic colorants used to help characterize the madder dye decolorization process, where R = cyclohexyl is C.I. Acid Blue 62.

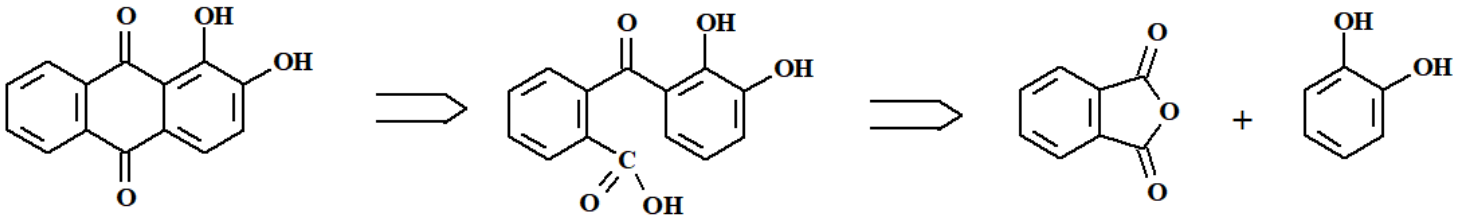

Figure 8

Retrosynthetic pathway associated with madder dye anthraquinone ring formation and subsequent Cl2-mediated degradation.

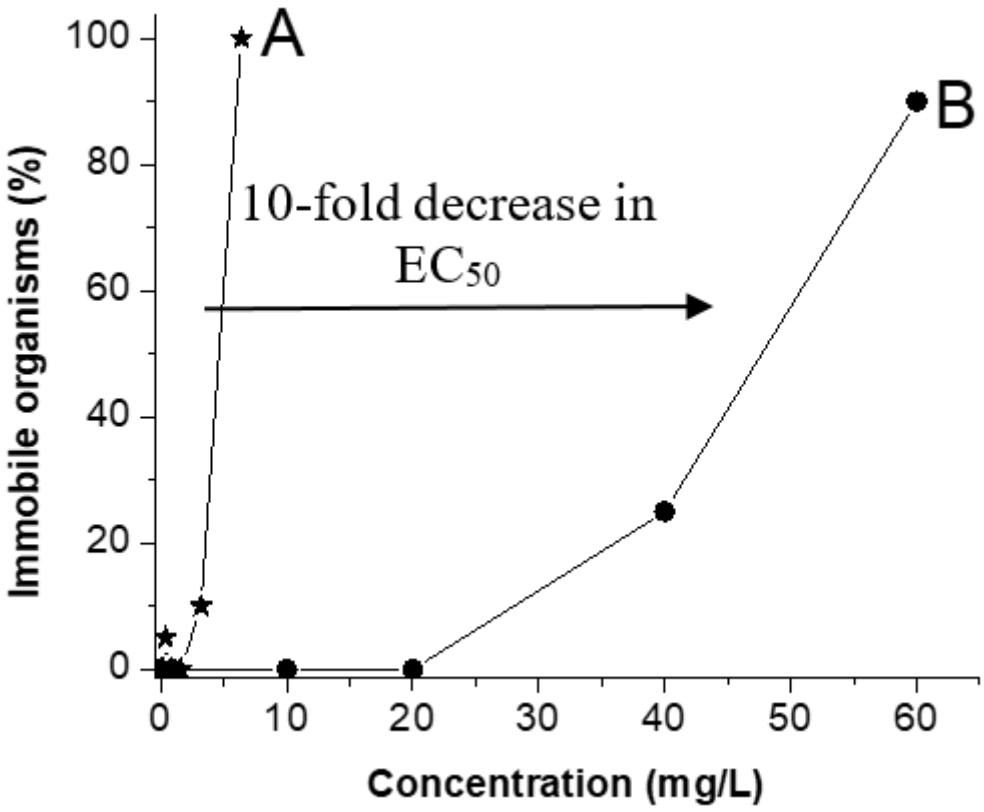

Figure 9

Relationship between the percentage of immobile D. similis and solution concentration for (A) madder and (B) chlorinated madder solutions.